Title

Association of Melanoma-Associated Antigen-A and Program-death ligand 1 Expression and Clinical Outcomes in Urothelial Carcinoma

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Association of Melanoma-Associated Antigen-A and Program-death ligand 1 Expression and Clinical Outcomes in Urothelial Carcinoma

A thesis submitted in partial satisfaction of the requirement for the degree Master of Science in Clinical Research

by

Izak Faiena

2019

ABSTRACT OF THE THESIS

Clinical Outcomes with Melanoma-Associated Antigen-A and Program-death ligand 1 Expression in Urothelial Carcinoma

by

Izak Faiena

Master of Science in Clinical Research

University of California, Los Angeles, 2019

Professor Robert M. Elashoff, Chair

Abstract:

Background: The melanoma-associated antigen-A (MAGE-A) and program-death ligand 1 (PD-L1) are present in urothelial carcinoma (UC). We aim to assess survival outcomes in patients with MAGE-A and PD-L1 expression.

Methods: Analysis of MAGE-A and PD-L1 expression on neoplastic cells was conducted using tissue microarrays from patients with UC. We compared differential expression between stage and grade. Co-expression of MAGE-A and PD-L1 were sub-categorized. Fisher's exact test was done for categorical variables followed by analysis of univariable and multivariable assessment of recurrence and progression free survival (RFS, PFS).

Results: Co-expression of MAGE+/PD-L1+ had a higher expression in advanced disease, however only MAGE+/PD-L1- group was associated with shorter RFS (HR 1.89, 95%CI 1.19-2.99; *p*=0.006). MAGE+/PD-L1+ was associated with the worst PFS (HR 17.1, 95%CI 5.96-49.4; *p*=<0.001). MAGE-A expression was more prevalent high-grade (p=0.015), and higher stage ≥pT2 (p=0.001). The 5-year RFS in MAGE+ was 44%vs.58% for MAGE- samples. On multivariable analysis MP was also associated with shorter recurrence (HR 1.55, 95%CI 1.05-2.30; *p*=0.03). Similarly, MAGE+ was associated with shorter PFS (HR 3.12, 95%CI 1.12-8.68; *p*=0.03).

Conclusion: We report that the expression of MAGE-A and PD-L1 is increased in more advanced disease and associated with shorter PFS. Furthermore, expression of MAGE-A is significant in higher grade and stage, and was associated with shorter RFS and PFS. The finding of worse prognosis of MAGE-A/PD-L1 provides early evidence that a potential combinatorial treatment strategy of co-targeting MAGE/PD-L1 with respective agents might be feasible. Further studies are needed to validate these findings.

The thesis of Izak Faiena is approved.

Allan J. Pantuck

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University of California, Los Angeles

2019

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List of Abbreviations

MAGE-A: Melanoma associated antigen-A

MP: MAGE positive

MN: MAGE negative

OS: Overall survival

PD-L1: Program-death ligand-1

PN: PD-L1 negative

PP: PD-L1 positive

PFS: Progression-free survival

RFS: Recurrence-free survival

TCR: Adoptive T-cell therapy

TMA: Tissue microarray

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1. Introduction

The MAGE-A gene family consists of 12 MAGE-A genes located on chromosome Xq28. The function and biological role of MAGE-A proteins in cancer have not been completely elucidated; however, members of MAGE-A have been implicated in modulating the activity of E3 ubiquitin ligases on targets related to apoptosis and in the suppression of p53-dependent apoptosis. [1] MAGE has been a highly attractive target for cancer immunotherapy because of its broad representation in cancer tissues but restricted expression in normal adult tissues, namely, immune-privileged germ cells. Recent studies have shown significant expression of MAGE antigen in urothelial carcinoma (UC). [2-6] This observation has clinical implications as other studies have shown a poor-prognosis in MAGE-positive patients. [6, 7] Currently, there are numerous approaches targeting this antigen in the clinical setting including vaccines, and adoptive T-cell therapy (TCR). A recent phase I trial using a TCR targeting MAGE-A has shown a potential benefit. [8] The safety of this TCR across multiple tumor types is currently being evaluated in a phase I trial (NCT03139370).

The use of immune checkpoint inhibitors in UC has become an important salvage option with reasonable response rates for patients who progress on cytotoxic chemotherapy. [9] While MAGE-A expression has been described in UC, evidence is lacking regarding the correlation of MAGE and PD-L1 expression in UC. This data is of interest, as it informs a potential combinatorial therapeutic strategy using adoptive cell transfer together with a checkpoint inhibitor. It is widely recognized that the use of CAR-T cells in solid oncology will likely require combination therapy

to address an immunosuppressive tumor microenvironment, with checkpoint inhibitors, for example. [10] Some early data suggest that a combination approach may potentiate an immune response and enhance the efficacy of using adoptive cell therapy. [11-13] In addition, there are studies that suggest that receiving checkpoint inhibitors prior to tumor infiltrating lymphocytes (TIL) harvest may lead to more effective TIL harvest, with a shorter *ex vivo* expansion time, and increased efficacy. [14, 15] In this study, we aimed to assess the survival outcomes in patients with urothelial carcinoma and the correlation with MAGE and PD-L1 expression.

2. Methods and Materials:

2.1. Study population and outcomes

The study cohort consisted of 422 UC samples in 275 patients from transurethral resection of bladder tumor or radical cystectomy done at a tertiary medical center between 1985 and 1998. Available clinical, pathological and follow-up data on each patient were obtained. The main covariates were age, gender, race, smoking history, cancer history, procedure, pathologic stage, and grade. The study outcomes were recurrence-free, progression-free survival in patients who underwent both RC and TURBT, and overall survival in patients who underwent RC only. The overall aim was the association of both MAGE expression with survival outcomes as well as MAGE and PD-L1 expression with survival outcomes.

2.2. Tissue microarray construction

Tissue from formalin fixed, paraffin embedded specimens were obtained. Three 0.6 mm core biopsies were taken from representative tumor regions and precisely arrayed using a custom-built

instrument as previously described. [16] Sections of four microns of the tissue microarray block were transferred to glass slides using the paraffin sectioning aid system comprising adhesive coated PSA-CS4x slides, adhesive tape and an ultraviolet lamp (Instrumedics, Hackensack, New Jersey) to support the cohesion of 0.6 mm array elements.

2.3. Immunohistochemical staining and evaluation

IHC analysis of MAGE-1 (mouse clone 6C1, Thermo Scientific), and PD-L1 (rabbit clone SP142, Spring Biosciences) was performed at room temperature (RT) on the Dako Link Autostainer 48 (Agilent, Santa Clara, CA). Tissue sections underwent pretreatment using Rip Tide, a proprietary antigen retrieval buffer (Mosaic Laboratories, Lake Forest, CA) for 40 minutes at 95°C. Once the Autostainer procedure was initiated, the slides were rinsed with buffer immediately and after each of the following steps: 1) incubation with Envision Peroxidase (Dako) for 5 minutes to quench endogenous peroxidase; 2) incubation with MAGE-1 antibody, PD-L1 antibody or isotype negative control for 30 minutes; 3) detection with Envision FLEX Linker for 15 minutes; 4) detection with Envision FLEX HRP for 20 minutes; and 5) staining with DAB (Dako) for 10 minutes each. Upon completion of the staining procedure, slides were counterstained offline with hematoxylin (Dako) for 2 minutes, rinsed and coverslipped.

Evaluation of IHC stains was performed by a pathologist who recorded the staining intensity, subcellular localization, and percentage of positively-stained tumor cells. Staining intensity was evaluated on a semi- quantitative scale with the percentage of cells staining at each of the following four levels recorded: 0 (unstained), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong

staining). An H-Score was calculated based on the summation of the product of percent of cells stained at each intensity using the following equation: (3 x % cells staining at 3+) + (2 x % cells staining at 2+) + (1 x % cells staining at 1+). The maximum staining intensity of normal adjacent tissue (NAT), endothelia, smooth muscle, fibroblasts, stroma, inflammatory cells, and nerve were recorded if observed. If positive PD-L1 staining was observed in endothelial cells, the percentage of staining was estimated. MAGE positive (MP) status was defined as ≥50% positive staining with 2+ or 3+ intensity as compared with MAGE negative (MN), a cut-off that has been used previously in evaluating MAGE staining. [8] A commonly used PD-L1 cutoff criteria of ≥1% positive staining (PP) of tumor cells was applied. [17]

2.4. Statistical analysis

Descriptive statistics for study variables were computed for the overall cohort as well as for the MP and MN subgroups. Study variables were compared between these subgroups using the Wilcoxon rank-sum tests for continuous variables and Chi-square or Fisher's exact tests for categorical variables. We further subdivided patients according to MAGE and PD-L1 staining status to assess the effect of co-expression on survival. Patients' specimen-level data was the unit of analysis for recurrence, as biopsies were done at each recurrence thus reflecting this particular outcome with follow-up time calculated from procedure until event or censoring. However, patient-level data was used to assess progression-free survival and overall survival. Recurrence-free survival curves were created with the Kaplan-Meier method and survival was compared between discrete MAGE and PD-L1 groups with the log-rank test. For recurrence, univariable and multivariable mixed-effects Cox proportional hazards model were used to evaluate predictors of

outcome, adjusting for repeated measurements per patient. Variables selected in the model were either significant on the unvariable and/or were felt to be clinically relevant. However, due to small number of events for progression, only a univariable analysis was conducted. Tests for proportionately were not violated in all models. Overall survival (OS) was analyzed only in patients who underwent radical cystectomy using Cox models. Performance of the Cox models was assessed using Harrell's C statistic. [18] A sensitivity analysis was also performed to assess effect of year of treatment on overall survival. The institutional review board approval at our institution was obtained. Two-tailed p-values <0.05 were considered statistically significant. Statistical analyses were performed with Stata statistical software version 15 (StataCorp, College Station, TX).

3. Results:

3.1. Patient characteristics

The patient cohort consisted of 275 patients and 422 samples (table 1 and 2). Median age was 70 (IQR 62-76) with the majority of the cohort consisting of male, Caucasian patients. A large proportion of patients were smokers or former smokers (54%). MAGE staining was associated with more advanced stage (≥pT2 52% vs. 42%), and high grade disease (73% vs. 60%; p=0.015). A similar trend was noted for PD-L1 positive samples, which were more likely being higher stage and grade, respectively.

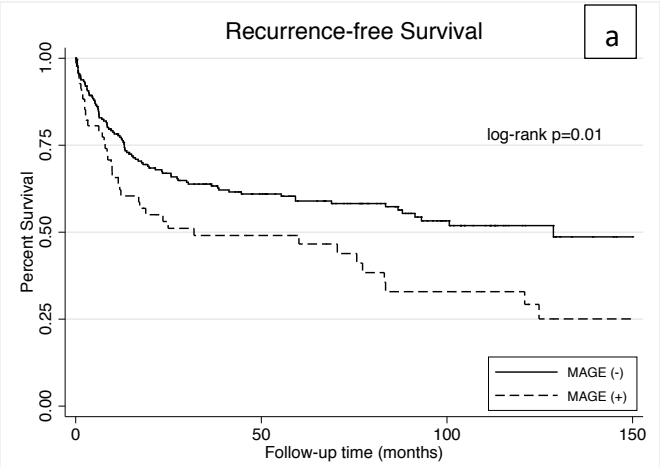
Table 1. Baseline characteristics	
	Total
	N=275
Age	70 (62-76)
Gender	
Male	223 (81)
Female	52 (19)
Ethnicity	
Caucasian	235 (85)
AA	6 (2)
Other	34 (12)
Tobacco	
No	29 (11)
Yes	149 (54)
Unknown	97 (35)
Personal History of Other Cancers	
No	148 (54)
Yes	52 (19)
Missing	75 (27)
Procedure	
TURBT	120 (44)
RC	136 (49)
Other	19 (7)

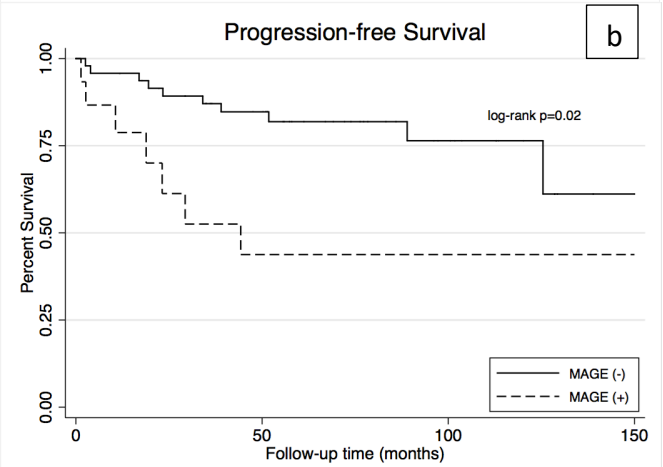
Table 2. Tumor and clinical characteristics - MAGE					MAGE and PD-L1 subgroups				
	MAGE Neg	**MAGE Pos**	**p-value**		**MN/PN**	**MP/PN**	**MN/PP**	**MP/PP**	**p-value**
Overall, n (%)	321 (76)	101 (23)			232 (55)	72 (17)	89 (21)	29 (7)	
Target Stage			<0.001						<0.001
pTa	113 (36)	21 (21)			96 (42)	17 (24)	17 (19)	4 (14)	
pTis	34 (11)	4 (4)			27 (12)	3 (4)	7 (8)	1 (3)	
pT1	38 (12)	23 (23)			25 (11)	17 (24)	13 (15)	6 (21)	
pT2	61 (19)	33 (33)			35 (15)	20 (28)	26 (30)	13 (45)	
pT3-4	50 (16)	13 (13)			30 (13)	10 (14)	20 (23)	3 (10)	
Metastasis	21 (7)	6 (6)			16 (7)	4 (6)	5 (6)	2 (7)	
Grade			0.015						<0.001
Low	129 (40)	27 (27)			109 (47)	24 (33)	20 (22)	3 (10)	
High	192 (60)	74 (73)			123 (53)	48 (67)	69 (78)	26 (90)	
Surgical margins			0.92						0.72
Neg	130 (40)	42 (42)			90 (39)	29 (40)	40 (45)	13 (45)	
Pos	13 (4)	3 (3)			10 (4)	1 (1)	3 (3)	2 (7)	
Missing	178 (55)	56 (55)			132 (57)	42 (58)	46 (52)	14 (48)	

3.2. MAGE outcomes

The median follow-up time for the entire cohort was 77 months (IQR 22-118 months). The median RFS for MP samples was 32 months, while those who were MN did not reach median RFS. The 5-year RFS in MP was 44% compared with 58% for MN samples (figure 1). On univariable cox model (table 3), MP was significantly associated with recurrence (HR 1.84, 95%CI 1.09-3.09; p=0.02), similarly on multivariable analysis adjusting for baseline and clinical variables, MP was also associated with shorter recurrence (HR 1.55, 95%CI 1.05-2.30; p=0.03). Model performance using Harrel's C statistic was 0.64. Median follow-up for patients who underwent RC was 38 months (IQR 13-101). The 5-year PFS in MP was 44% compared with 82% for MN samples (figure 1). On univariable analysis MP was also significantly associated with shorter PFS (HR 3.12, 95%CI 1.12-8.68; p=0.03). The median overall survival in patients who underwent RC and had MP staining was 46 months compared with 77 months in MN patients. In addition, 5-year OS was 44% vs 51% in MP vs. MN patients, respectively (figure 2). However, there was no association with OS in either the univariable (HR 1.15, 96%CI 0.71-1.87; p=0.56) or multivariable cox model (HR 1.01, 95%CI 0.58-1.75; p=0.97). Furthermore, in a sensitivity analysis, year of treatment did not influence OS.

Figure 1. Recurrence-free (A) progression-free (B) and overall survival (C) outcomes for MAGE-A staining (cutoff ≥50% positive staining with 2+/3+ intensity)





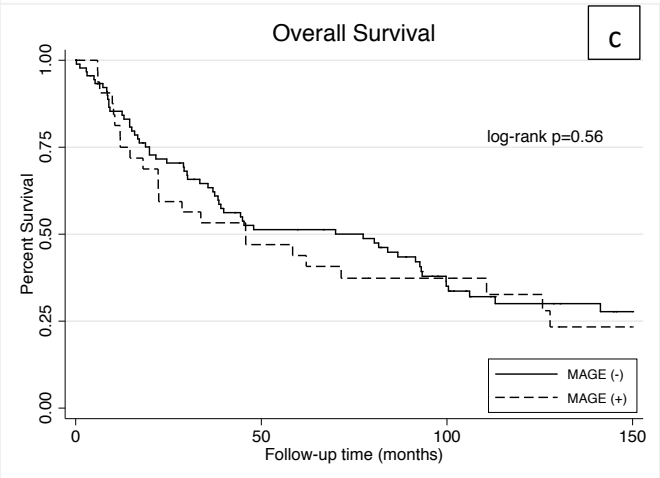
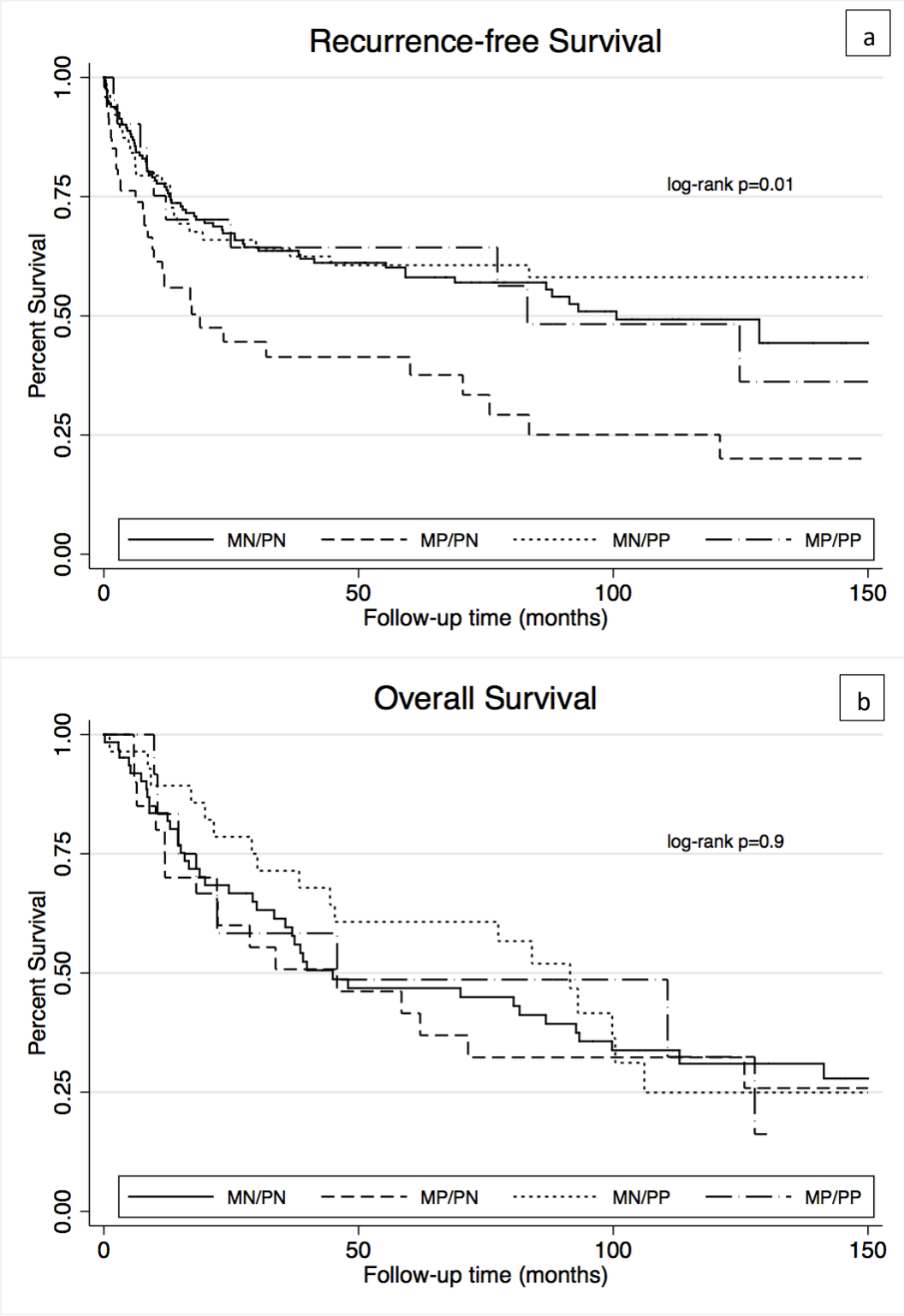


Figure 2. Recurrence-free (A) and overall survival (B) outcomes for MAGE-A (cutoff ≥50% positive staining with 2+/3+ intensity) and PD-L1 co-expression (cutoff ≥1% staining)



3.3. MAGE/PD-L1 outcomes

There were 27% PD-L1 positive samples. Co-expression of MAGE and PD-L1 was assessed by categorizing samples in subgroups by staining result (table 2). A higher proportion of non-muscle invasive bladder cancer samples (65%) were negative for both MAGE and PD-L1, whereas, there was a significant trend towards higher stage disease (p=<0.001) for samples that were positive for either marker. In the MAGE positive/PD-L1 negative group, 48% were ≥pT2, whereas in the MAGE negative/PD-L1 positive and double MAGE and PD-L1 positive groups, the percentages were 59% and 62%, respectively. A similar trend was observed in high grade disease; 90% of samples that were positive for both MAGE and PD-L1 were high grade (p=<0.001), for example. On univariable analysis, overall PD-L1 positivity was not associated with shorter RFS (HR 0.79, 95%CI 0.55-1.14; p=0.21). However, PD-L1 positivity was associated with shorter PFS (HR 5.31, 95%CI 1.99-14.2 p=0.001) (table 4). MAGE positive/PD-L1 negative samples comprised a subset associated with a shorter median recurrence-free survival of 19 months (HR 1.96, 95%CI 1.30-2.95; p=<0.001) (figure 2). Furthermore, when adjusting for clinical variables, this subset was still associated with shorter RFS (HR 1.89, 95%CI 1.19-2.99; p=0.006) (table 5). Model performance using Harrell's C statistic resulted in an AUC of 0.65. Of the associations observed, the most significant was the double-positive subset with shorter PFS (HR 17.1, 95%CI 5.96-49.4; p=<0.001). There was no difference in RFS among the other groups (table 4). OS was also not significant among the groups (figure 2).

Table 4. Progression-free survival model		
	Unadjusted	
	HR (95% CI)	p-value
MAGE		
Negative	Ref.	
Positive	3.12 (1.12-8.68)	0.03
PD-L1		
Negative	Ref.	
Positive	5.31 (1.99-14.2)	0.001
Groups		
MN/PN	Ref.	
MP/PN	4.16 (0.86-20.1)	0.08
MN/PP	6.62 (1.99-22.0)	0.002
MP/PP	17.1 (5.96-49.4)	<0.001

Table 5. Recurrence-free survival model – MAGE/PD-L1				
	Unadjusted		Adjusted*	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Groups				
MN/PN	Ref.		Ref.	
MP/PN	1.96 (1.30-2.95)	0.001	1.89 (1.19-2.99)	0.006
MN/PP	0.88 (0.56-1.38)	0.57	0.96 (0.59-1.55)	0.86
MP/PP	1.07 (0.58-1.99)	0.82	1.04 (0.52-2.07)	0.92
* Adjusted for age, gender, race, smoking history, pathologic stage, grade, and margins.				

4. Discussion

The treatment of urothelial carcinoma has undergone major changes in recent years, namely the addition of immuno-oncology agents that have been shown to provide significant benefit in advanced disease. [19] While the excitement is warranted, curative therapies for advanced disease are lacking. Cancer-testis antigens (CTAs), specifically MAGE-A, have emerged as potential targets for immune-oncologic strategies in the setting of advanced cancers. The prognostic implication of MAGE-A expression has been established in numerous malignancies without evidence of expression in normal tissue. [20] In UC, a number of studies using different expression analyses have shown that a significant proportion of patients with UC have expression of MAGE-A, and increased expression of MAGE-A was associated with shorter clinical outcomes. Dyrskjøt et al. have shown using an q-RT-PCR that 43% of MAGE-A expression was associated shorter

progression-free survival (HR 2.96, 96%CI 1.14–7.68; p=0.026). [4] Similar expression profile of MAGE-A was shown using TMA in a large cohort, which was associated with shorter cancer-specific survival in UC (HR 1.44, 95% CI 1.05–1.99, *p*=0.02). [6] In our study, using a more restrictive definition of MP in IHC samples, we found increased expression in 23%, which was significantly associated with shorter recurrence-free survival on both univariable and multivariable survival model. In addition, PFS was significantly shorter in MP group. In an exploratory analysis, we also found 43% MAGE-A expression using a less restrictive definition reported in previous studies. Furthermore, overall survival was not significantly different between MP and MN groups. This may be partly due to comorbidities or various treatments received that affect overall survival.

The possibility of using a combination strategy to target tumors, unleashing the immune system by targeting MAGE positive tissue while simultaneously allowing the immune system to function unimpeded via PD-L1 blockade is an attractive one. [21] Such strategies are being evaluated in other malignancies and have shown a potentiated response in combination approach in mouse models, [11, 12] And, clinical trials using a combination of adoptive cell and checkpoint inhibitor therapy are underway (NCT03296137, NCT03287674, NCT03296137). Thus, further exploration of MAGE and PD-L1 expression patterns in UC is warranted as a foundation for such combination approaches. In our secondary analysis, we explored the prognostic implications of both MAGE-A and PD-L1 expression, and whether it informs such a strategy in UC. To assess co-expression, we sub-categorized expression groups. The MAGE positive/PD-L1 negative group was associated with shorter RFS compared with the other expression groups. The MAGE and PD-L1 double-positive group was significantly associated with shorter PFS, but was not associated with shorter RFS, despite the fact that 62% of samples in this group were ≥pT2 and 90% were high grade (table

2), features that would be expected to associate with recurrence. This may be partially explained by the fact that many recurrences may have occurred in lower stage patients, and MAGE-A and PD-L1 expression seems to be more prevalent in more advanced disease.

The strengths of this study are first, the ability to look at a relatively large sample of UCs, while looking at the recurrence and progression outcomes in MAGE-A and PD-L1 expression. Second, to our knowledge, there are no studies looking at prognostic implications of the co-expression of MAGE-A and PD-L1 in UC. While it is unclear whether expression of MAGE-A will influence PD-L1 expression or vice versa, it is clinically relevant that there is a reasonable proportion of patients that simultaneously express MAGE and PD-L1 perhaps supporting a possible combination strategy. In addition, uniform TMA construction, staining and interpretation was done to reduce potential variability. The limitations of this study include its retrospective nature with many potential confounders and risk profile of each patient, such as performance status and treatments received, which may affect the results.

5. Conclusion

In this study, we demonstrate the association of MAGE-A IHC expression in UC with shorter recurrence and progression-free survival. Furthermore, co-expression of MAGE-A and PD-L1 are present in more advanced disease states, which translated into shorter PFS. This supports possible co-targeting of MAGE and PD-L1 strategy in advanced UC. Further study and validation of these findings are warranted.

6. APPENDICES

6.1. Data Origin

The tissue microarray was originally constructed from a historical database from 3 surgeons at UCLA between 1984-1998. Clinical data was entered on the individual patients as the database was kept prospectively. However, data was not entered fully. In addition, some important variables such was systemic therapy was not full 6recorded. However, the data is robust compared with other the concurrent literature and the number of samples for the TMA construction is robust for a meaningful analysis, and therefore, we decided to use this database and tissue to assess survival outcomes for MAGE and PD-L1 staining.

6.2. Statistical Considerations

6.2.1. Missing data

Initially, the question of missing data needed to be addressed, as we had an important variable of systemic therapy was missing in >70% of the data set. The reason is multifactorial. One reason is that most patients were not advanced disease in which systemic therapy is not indicated. Furthermore, standard of care had changed overtime and thus there were many patients who did not receive modern regimen prior to 1990. Finally, there is high potential for data entry error present. In order to assess the effect of time on our data, we conducted a sensitivity analysis by creating a categorized variable of YEAR 1984-1990 and >1990 and compared the survival outcomes, which did not show a difference. Other missing data were reduced significantly by using patient charts to add missing data on age, gender, race, stage and death. Data on cancer-

specific events was minimal, and we were unable to capture this data from prior records, which precluded the analysis of cancer-specific survival. After data entry there were still missing data, however at lower rate than prior to data cleaning. Missing data variables were included as separate variables in all models to adjust possible effects on the main outcomes.

6.2.2. Data set-up

The main challenge with TMA data is that there were multiple patients with multiple samples per surgical event, or multiple surgical events per patients. This presented a challenge regarding the analysis of this data. We initially attempted to use a multilevel hierarchal linear regression with level 1 as patient, and level 2 as the sample to assess the outcome of H-score, which was a numeric score for staining per sample. However, the model was not stable or easily interpreted as the H-score variable contained many values of 0, the linearity assumption was violated, as well as significant herteroskdasticity. In order to try to simplify the model, we attempted to categorize H-score to signify positive staining versus negative staining. We relied on prior publications for the definition of MAGE positive as well as PD-L1 positive staining based on percent and intensity of staining. Co-expression of MAGE and PD-L1 was assessed by grouping MN/PN, MP/PN, etc in order to evaluate the prognostic value of co-expression of MAGE and PD-L1 and whether this would inform a possible co-targeting strategy of MAGE and PD-L1 therapy. There was some thought to compare the continuous variable H-score between PD-L1 and MAGE however, there were multiple values of zero making a correlation more difficult. In addition, given that our definitions of positive vs. negative are becoming more commonly used in the literature, we felt that comparison using these definitions would yield meaningful results.

In order to further decrease the complexity of the data, multiple samples from one surgical event were averaged in order to have one data entry per surgical event. Patients also had multiple ancillary procedures coded (such as bowel resection, lymph-node dissection etc), which were categorized under the parent procedures of transurethral resection of bladder tumor, radical cystectomy and other (for procedures that did not fit the aforementioned defintions). Furthermore, patients had multiple similar surgical procedure (TURBT) or different procedures at different time points (TURBT followed by RC). The handling of procedure is described below for the survival analysis.

6.2.3. Baseline characteristics

Patient-level data was used to assess baseline characteristics including age, gender, race, smoking history, cancer history and procedure. For clinical characteristics, we decided to use sample-level data as it was felt that, for example, target stage made more sense to present on the sample level as a patient who had multiple procedures may have multiple different stages for each event thereby making difficult to describe a single stage per patient. We then compared staining groups (MP vs. MN and co-expression groups) using Fisher's exact test as there were a number of cells that had values fewer than 5. Continuous data, mainly age, was compared using Kurskal-Wallis test as the variable was not normally distributed.

6.2.4. Survival analysis

There were three main survival analyses done, recurrence-free, progression-free, and overall survival. The main challenge is to analyzing patient vs. sample-level data for recurrence. We decided to use sample-level data as the justification for this was that each surgical event, and staining, may inform possible future recurrence. For example, lower stage samples are known to recur frequently, and thus an initial surgical event with stage Ta, for example, may recurs on the next surgical event. The staining of the first event may inform possible future recurrences, and so we felt that this would give important information regarding this process. Similarly, for progression; Does a sample from a surgical event with a certain staining pattern predict a progression to a higher stage? However, for overall survival, there can only be one death event, and thus we used only patient-level data. Furthermore, this analysis was conducted in only patients who underwent radical cystectomy as those patients who will likely die of their disease. TURBT can be thought of more of a biopsy in lower stage disease and thus was not used for the purpose of overall survival.

The analysis was conducted using sample-level data to assess survival using the Kaplan-Meir method and log-rank test. For recurrence and progression, we used a mixed-effects Cox-regression model in order to assess the hazard ratio for univariable and multivariable analysis. The mixed-effects model was done in order to adjust for clustering of multiple samples per patient, and thus we introduced a mu term in the equation. Variables that were statistically significant in the univariable, as well as variables that were felt to be important clinically (such as stage and grade) were included in the multivariable model. We then assessed the proportionality of the models using Schoenfeld residuals and the assumption was not violated. Furthermore, to assess all model performance, we used Harrell's C statistic, which resulted in a

reasonable value of 0.64-0.65 for the models. A similar procedure was done for the analysis of overall survival, however, we used the standard Cox-regression model, as a mixed-effects adjustment was not necessary given one event per patient.

6.2.5. Additional results

In order to assess agreement of sample-level and patient-level data we evaluated the number of patients who had multiple biopsies one of which was MP and one MN. We found that all patients who had multiple biopsies had agreement. In other words, patients who had one biopsy MN and had a subsequent biopsy that was also MN, and vice versa.

In our progression-free survival analysis, we performed KM curve analysis on patient groups and is presented below. The log-rank test was statistically significant p=<0.001.

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